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Euphytica, (1995) Vol. 85, No. 1-3, pp. 119-123.

Thank-you!

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Euphytica 85: 119–123, 1995.

Biolistic introduction of a synthetic *Bt* gene into elite maize

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Key words: Bacillus thuringiensis, maize, microprojectile bombardment, transformation

Summary

A synthetic *Bt* gene encoding a truncated version of the CryIA(b) protein derived from *Bacillus thuringiensis* was successfully introduced into elite maize using microprojectile bombardment of immature embryos. The method used to initiate and identify transformation events is described. We describe the detailed parameters used for the Biolistics device as well as the plasmids used for the transformations. The plasmids contained the synthetic *Bt* gene driven by either the 35S CaMV promoter or a combination of two tissue-specific promoters, leaf and pollen, derived from maize. Specific conditions for the culture of Type I callus from immature embryos, the phosphinothricin (PPT) selection protocol, and the regeneration of plants are discussed. T0 and T1 plants were initially identified using the pH-dependent chlorophenol red test and/or the histochemical β-glucuronidase (GUS) assay. PCR and Southern data confirm the presence of the 35S CaMV promoter and the synthetic *Bt* gene.

Introduction

Cereal transformation via biolistic particle bombardment, and maize transformation in particular, remained elusive until recently (Potrykus, 1990). This report details the successful transformation of maize via microprojectile bombardment of immature embryos of an elite inbred. Transformants are recovered from Type I callus (Tomes, 1985) initiated after bombardment of the embryos. Other successful reports of maize transformation utilized suspension cultures (Fromm et al., 1990; Gordon-Kamm et al., 1990) or callus cultures of A188 × B73 crosses (Genovesi et al., 1992; Walters et al., 1992; Songstad et al., 1992).

Materials and methods

Source tissue

In 11 experiments, more than 2000 immature embryos of CG00526, a Lancaster-type maize inbred, were aseptically excised 14–15 days after pollination from surface-sterilized ears of greenhouse-grown plants. In each experiment, embryos, ranging in size from 1.5 to 2.5 mm in length, were cultured with the scutellum uppermost, on callus initiation medium, 2DG4 + 5 mg l^{-1} chloramben (Schweizerhal Inc., South Plainfield, NJ). 2DG4 medium is Duncan's (Duncan et al., 1985) 'D' medium modified to contain 20 mg l^{-1} glucose.

Preparation of DNA-coated microcarrier

Plasmid DNA was precipitated onto 1 μm gold microcarriers as described by the DuPont Biolistic manual (DuPont, 1990).

Two plasmids contained the synthetic *Bt* gene encoding for the CryIA(b) protein (Fig. 1) (Koziel et al., 1993). Plants from two transformation events were chosen for further study and for field evaluation. The two events, 171 and 176 were obtained using the synthetic *Bt* plasmids pCIB4431 or pCIB4418 (Fig. 1). The selectable marker for both events was supplied on pCIB3064 which carries a phosphinothricin acetyl transferase (*bar*) gene driven by the 35S promoter (Koziel et al., 1993). The DNA/gold mixture was prepared so as to deliver approximately 1 μg of

Fig. 1. Plasmid maps.

DNA per bombardment. The 171 event also contained pCIB3007 to encode the *gus* gene (Fig. 1).

Bombardment

Thirty-six embryos on a plate were bombarded using the Biolistics® PDS-1000HE device (DuPont, Wilmington, DE) according to the manufacturer's protocol. The tissue was placed on the shelf 8 cm below the stopping screen shelf. A 10 × 10 μm stainless steel screen hand-punched at Ciba or a 24 × 24 μm standard screen supplied by the Biolistic manufacturer was used with rupture discs of 1550 psi value for the bombardments. Following bombardment, the embryos were cultured in the dark at 25° C.

Callus formation

One to 14 days after bombardment, embryos were transferred to callus initiation medium containing 1–3 mg l^{-1} phosphinothricin (PPT) and incubated in the dark at 25° C. Embryos were scored for Type I embryogenic callus formation at 2 and 3 weeks after bombardment. Embryogenic tissue was transferred to callus maintenance medium, 2DG4 + 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) containing 1–3 mg l^{-1} PPT. Type I callus was subcultured every two weeks for a total of 12 weeks to fresh maintenance medium containing 3–10 mg l^{-1} of the selection agent.

Regeneration

Regeneration from the selected callus was initiated after 12 weeks on PPT. Type I callus was subcultured onto a modified Murashige & Skoog medium (MS) (Murashige & Skoog, 1962) containing 3% sucrose, 0.25 mg l^{-1} 2,4-D and 5 mg l^{-1} benzylaminopurine and cultured under 16 hours of light (50 μE $m^{-2}s^{-1}$), 8 hours dark, at 25° C. Two weeks later, the tissue was transferred to MS medium containing 3% sucrose without phytohormones. Plants regenerated during the following 4–10 weeks. Regenerated plants were cul-

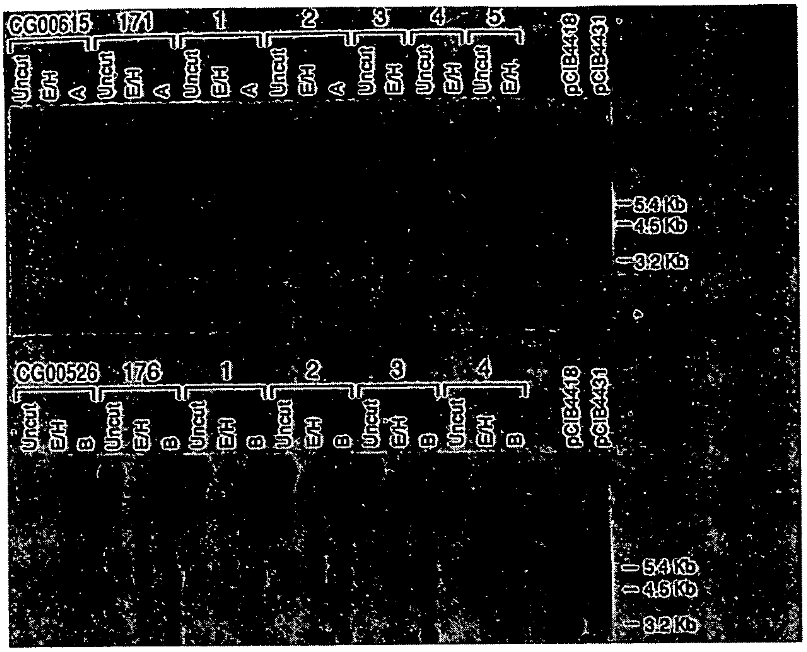

Fig. 2. Southern analysis of 176 event and progeny.

tured on MS medium modified to contain half the concentration of salts and 3% sucrose.

Assays for verification of transformation

Multiple leaf pieces from all plants were first tested histochemically for GUS expression (Jefferson, 1989), if applicable, and expression of the *bar* selectable marker gene using the chlorophenol red test (CR) (Kramer et al., 1993). In this assay, tissue that is resistant to PPT can actively grow on medium containing the compound and can acidify the pH of the medium which is indicative of healthy tissue. Within 4–7 days, resistant tissue changes the medium colour from deep red to bright yellow. PCR analysis (Perkin Elmer Cetus, 1991) followed the GUS and CR assays, and plants positive in these assays were transferred to the greenhouse for insect testing (Koziel et al., 1993) and maturation. Several plants were also tested by ELISA (Clark et al., 1986) for the synthetic *Bt* protein. Southern blot analysis was also done using standard methods (Sambrook et al, 1989), see Fig. 2.

Embryo rescue

To meet a field planting deadline, traditional harvesting methods could not be used. Embryos from T1 progeny transformed plants were 'embryo rescued' (Weymann et al., 1993). Fourteen to 16 days after pollination, the ear tip with 25–50 kernels was cut from the ear with a coping saw. The excised ear piece was surface-sterilized and individual embryos were excised and plated with the scutellum uppermost on B5 medium (Gamborg, 1968) containing 2% sucrose. Rescued seedlings were tested for the presence of the inserted genes in the same manner as the transformed parent.

Results

Table 1 lists the eleven experiments that yielded maize transformants when immature embryos of CG00526 were bombarded. Thirty-two percent of the total 2000 treated embryos initiated Type I callus, e.g., for the 171 event, 18% of the embryos bombarded produced Type I callus; for the 176 event, the callus initiation frequen-

Table 1. Experiments and bombardment conditions from which transformation events were recovered

Expt.	Embryos plated	Responding embryos	Events recovered	Selection conditions[1]	Plants regenerated
1	235	65	7	3/14	1355
2	129	64	1	1/5→3/35→10/63	251
3	102	59	1	1/3	171
4	36	19	1	1/3→3/33→10/60	465
5	140	80	4	1/3→3/33→10/60	2322
6	137	86	1	3/1	1676
7[2]	247	60	2	3/1	693
8[3]	249	44	2	3/1	515
9	251	37	1	3/1	727
10	162	46	1	3/3	679
11	318	86	2	3/3	6460
Total	2006	646	23		15314

[1] mg l^{-1} PPT day^{-1} applied after bombardment.
[2] Event 171 recovered.
[3] Event 176 recovered.

cy was 15%. Every embryo which produced callus was cultured separately thus representing an individual transformation event. Selection was carried out using 1–3 mg l^{-1} PPT at 1–14 days after bombardment. Since these were early experiments, the optimal selection protocol had not been determined (Table 1). A total of 15,314 plants were regenerated from the 11 experiments. All of the plants recovered were assayed by the CR test for the presence of the *bar* gene and by histochemical analysis for *gus* gene expression, if applicable. Positive plants identified by these assays were analysed by PCR for the presence of the 35S promoter and/or the *Bt* gene. Plants that assayed positive in all these tests were subsequently assayed by ELISA for the *Bt* protein and subsequently fed to insects.

Two events, 171 and 176, were chosen from the positive events for further development. Thirty-three plants were recovered from the 171 event and 38 from the 176 event. Plants that assayed positive in the CR and GUS (if applicable) assays were in turn assayed by PCR analysis for the presence of the 35S promoter on the selectable marker and the synthetic *Bt* gene. Plants that were positive by PCR assay were moved to the greenhouse for insect bioassay and maturation. For the 171 event, 25 plants were positive and 8 negative by the CR assay. All positive plants expressed the *gus* gene. For the 176 event, 8 plants were positive and 30 were negative. In eleven experiments, a total of twenty-three independent events were recovered or an average of two independent events per experiment.

All transformants were fertile, producing abundant pollen and full ears. Plants were backcrossed and outcrossed to several Ciba Seeds elite inbreds.

In some cases, embryo rescue was employed to produce the T1 hybrid seedlings for planting in the field. Leaf samples were taken from the seedlings for analysis by GUS histochemical assay, PCR analysis, CR assay, ELISA for CryIA(b) protein and insect bioassay with European corn borer (ECB) larvae. After analysis, almost 1,000 plants were shipped to the field.

Discussion

The results presented in this paper demonstrate that maize transformation can be routinely accomplished using immature embryos as the source tissue. In this study, independent transformants were recovered from 11 experiments, multiple transformation events were recovered from single experiments, and multiple plants were recovered from each event.

Interestingly, a range of culture parameters can be employed to achieve maize transformation via microprojectile bombardment of immature embryos. The selection agent (PPT) can be applied from 1 to 3 mg l^{-1} from 1 to 14 days after bombardment. Transformants

can be recovered from 'step-up' as well as constant-level selection pressure.

It is possible that a range of bombardment conditions such as rupture disk pressure and distance to the target may also be applied. The success of this maize transformation procedure is likely to be due, in part, to the initial age and general good health of the immature embryos as well as the subsequent culture maintenance of the lines under selection pressure. Using a genotype with a proven history of high callus initiation frequency, simplifies the procedure.

Further, the use of embryo rescue techniques enabled us to meet deadlines for the first field testing of elite maize engineered for insect resistance. This procedure saved 30 days in recovering T0 progeny (T1 plants) for field evaluation and allowed for testing during the field season in which the transgenics were produced.

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